Development and Characterization of Vascular Endothelial Growth Factor Fused with Elastin-Like Polypeptides for Chronic Wound Healing

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Abstract of the thesis:

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Chronic wounds are wounds that fail to go through the key stages of wound healing in a timely and orderly way. Previous research shows that chronic wounds could be a result of impaired blood flow to the wound, as seen in diabetic wounds and pressure ulcers. Around 6.5 million Americans are affected by chronic wounds and the US spends around 23 billion dollars on the treatment of these wounds each year. Current treatments are expensive, ineffective, and could have serious side effects. The use of growth factors is being increasingly investigated as a possible treatment for chronic wounds. However, the hostile environment in chronic wounds that contain a large number of proteases results in exogenous growth factors being quickly degraded within the wound environment. This limitation has prevented growth factors from becoming widely utilized as a treatment option.

This work, therefore, shows the development and characterization of vascular endothelial growth factor fused with elastin-like polypeptide (VEGF-ELP) fusion proteins, which have been hypothesized to remain stable for longer durations in the wound environment by resisting degradation by proteases. The protein was developed as recombinant fusion protein nanoparticles using transformation into a bacterial system and a protein purification technique called Inverse
Transition Cycling (ITC). Purity was assessed using techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. The physical characterization of VEGF-ELP was done using a turbidity test and particle size measurement on the purified final product. Several biological assays were also conducted in vitro to show that VEGF-ELP can enhance the different processes which play key roles in wound healing, such as cellular proliferation, migration, and angiogenesis. It was found that endothelial cells show increased proliferation and tube formation and that fibroblasts have better migratory characteristics in the presence of VEGF-ELP. Future steps could involve in vivo testing in experimental animal models with spinal cord injury and diabetes to demonstrate enhanced wound closure in the presence of VEGF-ELP.
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Chapter 1: Introduction

1.1 Stages of Wound Healing

Wound healing is a complex process that involves various cellular, molecular, and humoral responses [1]. Acute wound healing has four distinct stages that include clotting, inflammation, proliferation, and remodeling. Immediately after an injury to the skin, the first phase of healing is a cessation of bleeding through the initiation of various clotting cascades. The injured skin causes a release of clotting factors and thrombocytes, platelets aggregate, and a fibrin clot is formed to stop the bleeding of the wound [2]. The clot, which consists of fibrin, fibronectin, vitronectin, and thrombospondins, acts as a matrix to enhance the migration of fibroblasts, endothelial cells, and keratinocytes into the injured area during the proliferative stage of wound healing.

The first stage of wound healing is followed by the inflammatory stage, which occurs 2-6 days after the initial injury [1]. Damaged cells within the wound release certain molecules that attract inflammatory cells such as macrophages and neutrophils to the wound environment [3]. Neutrophils secrete mediators such as Tumor necrosis factor-α (TNFα), Interleukin-1 (IL-1), IL-6, which amplify the inflammatory response and stimulate secretion of growth factors, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) [4,5]. Neutrophils also secrete proteases, such as elastase and proteinase 3, which breakdown damaged tissues and proteins in the wound.

The inflammatory stage of wound healing is also characterized by an influx of macrophages into the injury zone. Macrophages have key functions such as ridding the wound of pathogens through their role as antigen-presenting cells, preventing infection, and removing apoptotic cells [6]. They also secrete a variety of growth factors such as VEGF, PDGF, and
fibroblast-derived growth factor (FGF) which aid in activating the next stage of healing by promoting cellular proliferation and extracellular matrix synthesis [7].

The third stage of acute wound healing is the proliferative stage, which usually occurs 3-10 days after injury. The key processes that occur during this stage of healing are proliferation and migration of different types of cells, restoration of a vascular network, and the formation of granulation tissue [1]. During this stage, fibroblasts migrate from surrounding tissues into the wound area and play a key role in increasing collagen deposition to form a new extracellular matrix. The development of the extracellular matrix along with the binding of essential growth factors, such as VEGF and PDGF, stimulates endothelial cell proliferation and migration. The endothelial cells also begin to synthesize new lumen and form capillary-like tube networks. These tubes lead to the formation of new vasculature within the tissue, which leads to increased blood flow, oxygen, and nutrient delivery to the wound [8,9,10].

The final step in the proliferative stage of wound healing is the formation of granulation tissue. Granulation tissue consists of fibroblasts, granulocytes, macrophages, collagen bundles, and it is highly vascularized [1]. This tissue replaces the provisional fibrin matrix that was formed during the first stage of wound healing [11]. Fibroblasts play a large role during this stage in the production of collagen and extracellular components such as fibronectin, proteoglycans, and hyaluronic acid [12].

The fourth stage of healing is the remodeling stage, which lasts from three weeks to a year after injury. During this stage, the extracellular components strengthen and the angiogenic processes reduce. Primarily, fibroblasts differentiate into alpha-smooth muscle actin expressing myofibroblasts, which are integral in forming stress fibers that allow contraction and wound
closure [13]. The remodeling stage concludes with the formation of scar tissue over the wound as collagen fibers are rearranged and aligned within the new dermal layers.

1.2 Chronic Wounds

Chronic wounds, in contrast to acute wounds, do not go through the four distinct stages of wound healing in a timely and orderly fashion [14]. They often get delayed in one of the earlier stages of wound healing, such as the inflammatory stage. Some of the consistent components that contribute to the formation of chronic wounds are bacterial colonization, repeated injury, cellular and systemic changes associated with aging, and tissue hypoxia [15]. Research provides evidence that the main contributing factor in the formation of chronic wounds is impaired blood flow to the wound [16]. Blood flow is vital to wound healing because oxygen is necessary to create the energy needed for various cellular processes such as cellular proliferation, migration, collagen synthesis, and immune responses [17].

Research shows that when normal wounds experience oxygen deficiency or hypoxia, there is up-regulation of growth factors that stimulate angiogenesis within the tissue so that increased blood flow can lead to increased oxygen delivery. However, in chronic wounds, hypoxia is unable to elicit an angiogenic response that is sufficient. This leads to insufficient amounts of oxygen and nutrient delivery to the wound, which prevents the wound from transitioning into the proliferative stage of wound healing.

The lack of oxygen to the wound causes a drop in ATP production due to reduced mitochondrial phosphorylation. This, in turn, causes failure of ATP dependent transmembrane proteins, which leads to disruption of the regular transport of key molecules across the cellular membranes [18]. One disrupted cellular process leads to the release of additional cytokines such
as TNF and IL-1, which attract more neutrophils and macrophages to the wound. These cells, as a result, generate excessive amounts of certain proteases, which leads to degradation of the necessary growth factors and extracellular matrix (ECM) components such as collagen and fibronectin [19,20]. The breakdown of these proteins leads to further promotion of the inflammatory stage of wound healing. Thus, a positive control loop is initiated, in which the wound is prevented from transitioning from the inflammatory stage of healing into the proliferative stage.

There are several reasons that a wound can experience inadequate blood flow. One reason could be that the patient is diabetic and has decreased peripheral circulation. Another reason could be due to some sort of injury, such as spinal cord injury, which affects blood flow to some parts of the body. Other reasons for tissue hypoxia could be arteriosclerosis, hypertension, or fibrosis [17].

The four main types of chronic wounds are venous ulcers, arterial ulcers, diabetic ulcers, and pressure ulcers. Venous ulcers are shallow and irregularly shaped and characterized by pigmentation in the surrounding skin. They are usually formed in the medial gaiter area of the leg [21]. Arterial ulcers are characterized by a thin, shiny appearance, and are usually painful. They are usually formed on the foot, toes, or heel. Diabetic ulcers are painless due to decreased sensation at the affected site and are usually formed on the foot. Pressure ulcers can be painful or painless and they may extend to the bone. They usually form on the lower half of the torso [21].

Some of the complications that arise due to these chronic wounds include infection, osteomyelitis, edema, hemorrhage, as well as the need for amputation of the limb. Each year, 6.5 million Americans are affected by chronic wounds. As a result, the US spends around 25 billion dollars each year on the treatment of chronic wounds [22]. These numbers are expected to rise as the incidence of obesity and diabetes increase in the population. Thus, it is apparent that chronic wounds constitute a significant healthcare problem in the US and throughout the world as well.
1.3 Current Treatments

Current treatments for chronic wounds range from surgical debridement to the use of growth factors. Surgical debridement involves the removal of necrotic tissue from the wound using surgical tools, enzymes, or maggots [21]. This method can be quite painful and cause extensive bleeding of the wound. Compression stockings are often used to increase circulation to the area of the wound. However, these tight wraps can obstruct blood flow to other areas of the body and can pose a clotting risk for some patients. Vacuum-assisted wound closure is the use of a vacuum to suck out excess wound fluid from the wound and to help bring the edges of the wound together. Some cons of this method are that it can limit patient mobility, can be painful, and there is limited clinical evidence to support its therapeutic efficiency [21].

One clinical instrument used to restore blood flow to the area of the wound is the hyperbaric oxygen chamber. Using this chamber, oxygen is supplied to the patient under high pressure to increase oxygen delivery to the wound by increasing the dissolution of gaseous oxygen in the blood. This method has been shown effective in about 50% of the cases in which the patient was not responding to other forms of treatment and would eventually require amputation. However, it is incredibly expensive, requires hospital care, and could also cause a range of serious side effects such as lung trauma and seizures due to oxygen toxicity to the body [23,24].

As discussed, current treatments lack the therapeutic efficacy needed to allow for complete healing. They fail to restore the molecular and cellular environment that is seen in acute wound healing and they can be ineffective, painful, and even harmful. Therefore, one treatment method that is being increasingly investigated for the treatment of chronic wounds is the topical application of growth factors. Growth factors are proteins that are natively found in the wound environment. They play roles in many of the processes that are required for wound healing, such as cellular
proliferation, migration, and restoration of the vascular networks. More research is required before growth factors can be widely used as a treatment option for chronic wounds.

1.4 Vascular Endothelial Growth Factor

This thesis project explores the use of VEGF as a treatment option for chronic wounds. VEGF plays a role in many of the processes that occur during wound healing, such as angiogenesis, collagen deposition, and epithelialization. Since tissue hypoxia is the key factor in the development of chronic wounds, VEGF could be instrumental in renewing vascular perfusion to a wound and restoring the healing process.

VEGF is a homo-dimeric glycoprotein with subunits linked by disulfide bonds [25]. It is produced by many of the key cells that are part of the wound healing process, such as endothelial cells, fibroblasts, platelets, neutrophils, and macrophages [25, 26]. There are 5 VEGF ligands; VEGF 165, VEGF 121, VEGF 145, VEGF 189, and VEGF 206. Each VEGF ligand is either bound to heparin on the cell surfaces or is freely secreted. VEGF 165 is found in equal amounts in the bound and free forms, which increases its bioavailability within the body. It also binds the largest number of cellular receptors, including the receptors that are most pertinent to wound healing. As a result, VEGF 165 is most often used for wound healing applications. In this research project, we use VEGF 165 to investigate the possible therapeutic value of VEGF in wound healing [27].

VEGF 165 binds to the VEGFR1 and VEGFR2 receptors, which are members of the tyrosinase kinase family. The analogous VEGFR-2 receptor in mice is important in endothelial cell differentiation [28]. It plays a role in regulating chemotaxis and proliferation of endothelial cells. The VEGFR-1 receptor is known to play a role in mediating the response of neutrophils and
macrophages, increasing vascular permeability, and inducing the expression of anti-apoptotic proteins [29].

VEGF is directly known for stimulating angiogenesis, through many receptor-mediated actions. It increases vasodilation, basement membrane degradation, and capillary tube formation by enhancing endothelial cell proliferation. Binding to the VEGF-R2 receptor stimulates nitric oxide synthase, which allows for the synthesis of nitric oxide, which is an effective vasodilator [30]. Vasodilation can increase endothelial cell response to the circulating growth factors within the wound environment and can induce more VEGF expression [21]. Degradation of the basement membrane allows for the movement of endothelial cells into the injured area. Migration and proliferation of endothelial cells, in turn, will lead to increased tube formation and renewal of vascular perfusion to the wound.

VEGF has many indirect effects on the wound healing process as well. It plays a role in increasing leakage of fibronectin and fibrinogen, mediating key ECM proteins, such as integrins, and increasing collagen deposition. These factors also contribute to aiding endothelial cells and fibroblasts in migrating into the wound. As discussed previously, the endothelial cells will renew the vasculature and the fibroblasts will differentiate into myofibroblasts and aid a contracture of the wound.

To summarize, VEGF plays a vital role in native wound healing. As discussed in previous sections, decreased blood flow to the chronic wound results in decreased oxygen and nutrient delivery which leads to a prolonged inflammatory period and reductions in cellular proliferation, migration, and angiogenesis. Thus, in a clinical setting, the application of VEGF to a chronic wound could potentially aid in promoting repair.
1.5 Limitation of Native VEGF

Although VEGF has a significant role in stimulating angiogenesis by increasing vascular permeability, regulating ECM components, and stimulating proliferation of endothelial cells, there is one key limitation in using native VEGF as a topical stimulator for enhanced wound healing. Native VEGF is quickly degraded within the wound environment by proteases. As discussed previously, chronic wounds have a high amount of protease activity due to the overstimulation of neutrophils, as seen during the prolonged inflammatory phase. These proteases circulate within the wound and degrade collagen, fibronectin, as well as essential growth factors. This is one of the factors for the delay in healing in chronic wounds. Thus, it has been shown that topically applied growth factors, such as VEGF are also quickly degraded in the hostile environment [30].

As a result of this quick degradation, a topical application of VEGF for the treatment of chronic wounds would need to be applied frequently and in high dosages for any therapeutic efficacy [30]. The wound would also have to be redressed frequently by patients or healthcare providers. This renders VEGF as an expensive and impractical treatment option for renewing vascular perfusion in chronic wounds. Repeated application of the topical ointment to the wound could also result in increased chances of malignancy and tumor formation, as the cells are being repeatedly exposed to external chemicals.

Due to this major limitation, VEGF is currently not available as a treatment option for chronic wounds despite its significant role in the wound healing process. The goal of this thesis is to address this limitation by offering a possible solution. A solution is required that would allow VEGF to remain active within the wound environment for longer periods by resisting the degradative action of the proteases within the wound. This would reduce the need for frequent application and would increase the therapeutic efficacy at a lower cost and risk.
1.6 Application of Elastin-Like Polypeptides

Elastin-like polypeptides (ELP) are derivatives of tropoelastin, consisting of repeats of Valine, Proline, Glycine, Xaa, and Glycine, where Xaa can be any amino acids other than proline. These proteins are nonimmunogenic, nonpyrogenic, and biocompatible [32]. ELPs are being increasingly investigated for biomedical applications due to the unique property that they transform from a soluble monomer to an insoluble nanoparticle over a certain transition temperature. This transformation is a reversible phenomenon; below the transition temperature, they will return to their soluble form. The transition temperature can be adjusted as it is a function of the ELP chain length, the Xaa residue, and the salt concentration in the sample during purification [33].

The main advantage of ELP’s is that the reversible transition from a monomer into a nanoparticle can be utilized during the purification process. Companies spend a lot of resources and budget on chromatography and filtration steps during the purification of proteins for therapeutic applications. By utilizing the unique property of the ELP, a method called inverse transition cycling (ITC) can be used to purify the proteins easily and inexpensively.

A second benefit of the ELP is that the transition temperature can be fine-tuned by adjusting the chain length and the identity of the Xaa sequence. As a result, we can create an ELP protein that has a transition temperature around 37°C so the protein forms nanoparticles when placed into the body. The formation of nanoparticles, in turn, protects the protein from degradation in vivo.

Thus, ELP’s offer a viable solution to the problem addressed in this thesis. As discussed, native VEGF is a critical factor in restoring the molecular and cellular environment of a chronic wound to that of a healing wound. However, VEGF is degraded within the hostile wound environment at a rate that makes it inefficient as a therapeutic option [30]. The fusion of VEGF with ELP offers a possible solution to this issue by allowing VEGF to resist degradation from
proteases through the formation of nanoparticles. This leads to the third benefit of using ELP, which is that it will allow for the VEGF to have a sustained release profile within the chronic wound. As a result, the VEGF should remain therapeutically active for longer periods, which will prevent the need for reapplication and could make it an efficient, practical, and inexpensive treatment option for chronic wounds.

1.7 Thesis Summary

This thesis work aimed to show that angiogenic VEGF-ELP fusion proteins could successfully be developed, characterized, and shown to be biologically active. The thesis workflow is shown in Figure 1. We hypothesized that there would be evidence that VEGF-ELP fusion proteins can enhance chronic wound healing and that they would be better equipped to withstand protease degradation due to nanoparticles formation. The hypothesis was tested with five specific aims, which as stated were:

1) To develop and isolate the VEGF-ELP fusion protein nanoparticles
2) To characterize the VEGF-ELP fusion protein nanoparticles
3) To assess the ability of VEGF-ELP to stimulate cellular proliferation in vitro
4) To assess the ability of VEGF-ELP to enhance cellular migration of fibroblasts in vitro
5) To assess the ability of VEGF-ELP to increase capillary-like tube formation in vitro

In Chapter 2, we developed a procedure to develop and purify the VEGF-ELP fusion proteins by using bacterial transformation, sonication, and ITC purification. These methods were optimized to yield a workable amount of protein, as determined by BCA assays. Temperature, centrifuge
speed, spin durations, and pH were all fine-tuned through repeated trials. SDS-Page and western blot results revealed that the VEGF-ELP fusion protein was successfully created and isolated.

In Chapter 3, we aimed to provide evidence of VEGF-ELP nanoparticle formation over a certain transition temperature. A turbidity test was used to show formation of nanoparticles through visual observation and measurement. It was revealed that nanoparticles were forming above a physiologically relevant temperature. After nanoparticle formation was demonstrated, the size of these particles was determined using dynamic light scattering (DLS).

In Chapter 4, we assessed the ability of the VEGF-ELP to enhance endothelial cell proliferation \textit{in vitro}. This was done using an MTS proliferation assay. It was found that VEGF-ELP enhanced cellular proliferation of endothelial cells to a much greater degree than the control and ELP groups. VEGF-ELP exhibited comparable stimulation of proliferation when compared to native VEGF.

In Chapter 5, we assessed the ability of the VEGF-ELP to enhance fibroblast migration \textit{in vitro}. A migration assay protocol was optimized to visualize and quantify fibroblast scratch closure over 48 hours. Results suggested that VEGF-ELP can enhance migration to a greater degree than the control or ELP.

In Chapter 6, we assessed the ability of the VEGF-ELP to enhance endothelial cell tube formation. Cells were seeded in matrices and imaged after 18 hours using a fluorescent microscope. Results were quantified based on the number of dots, tubes, and meshes in all groups. VEGF-ELP and VEGF showed a comparable formation of tubes and a significantly greater tube formation over the controls and ELP alone groups. The results suggested that VEGF-ELP can enhance tube formation.
Chapter 7 summarizes the results achieved from the various experiments and discusses the implications of this research in the treatment of chronic wounds. Limitations of this research and future steps to provide more evidence on the therapeutic value of these VEGF-ELP fusion proteins were also be discussed.

![Figure 1. Overall Experimentation Workflow](image)

Figure 1. Overall Experimentation Workflow. Depicts the steps developed as part of this thesis project, including development of the VEGF-ELP protein, purification, characterization, *in vitro* experiments, and future steps for the project.
Chapter 2: Development, Transformation, and Purification of VEGF-ELP Fusion Protein Nanoparticles

2.1 Introduction

One of the benefits of using ELP is that this protein acts as a purification tag due to its reversible formation of nanoparticles over a transition temperature [34]. In this chapter, we grew large quantities of the VEGF-ELP fusion protein by using bacterial transformation. We then used sonication and ITC to isolate the protein from the other bacterial components. The purity of the final product was assessed using SDS-PAGE and western blot.

2.2. Materials and Methods

2.2.1 Cloning and Expression of VEGF-ELP

Luria-Bertani (LB) media, Terrific Broth (TB) media, and LB with agar media were used to clone the transformation plasmid. VEGF-ELP was cloned using a pET25B+ plasmid with 50 pentapeptide repeats of ELP. The sequencing of this plasmid was done by GenScript. Three restriction enzymes were found: Mph1103I, NdeI, and HindIII. The ELP was connected to the VEGF chain using the NdeI and HindIII restriction enzymes. A histidine tag was also inserted at the N-terminal using the Mph1103I restriction enzyme. This tag can be utilized as a marker for identification in western blotting experiments. The final plasmid used for transformation is shown in Figure 2.

Transformation was done using a heat shock mechanism that works by creating pores in the bacterial host’s plasma membrane to allow for the insertion of the plasmid into the bacterial cell genome. E. coli (BL21 Star DE3) was used as the bacterial host and was obtained from
Invitrogen. 1 μL of the plasmid was added to 20 μL of the E. coli and kept on ice. The tube was then heated to 42°C in a water bath for 2 minutes to allow for bacterial uptake of the plasmid. SOC media (Novagen) was added to the tube containing the bacterial host and the plasmid and the solution was incubated at 37°C for 1 hour. A 50 mg/mL stock solution of carbenicillin was created for the next steps.

A petri dish was made using 25 mL of agar solution with the carbenicillin antibiotic (1:1000). After transformation was performed, 10 μL of the E. coli solution was spread on the agar plate using the streaking method. After 24 hours, there were many bacterial colonies growing on the agar plate. The final plasmid that was retransformed contained a gene for carbenicillin resistance. As a result, only bacterial cells that had incorporated the plasmid with the protein of interest were able to resist the carbenicillin on the agar and survive. Bacterial cells that did not incorporate the plasmid did not contain the carbenicillin resistance gene and were unable to survive on the agar and replicate.

One E. coli colony was picked from the agar plate and grown for 24 hours in media with carbenicillin (1:1000) in the incubator shaker (220 rpm). After another 24 hours, this bacterial culture in the LB media was used to inoculate 1 L of TB media with carbenicillin (1:1000). This culture was grown for 24 hours in the incubator shaker (220 rpm). After 16-18 hours, the sample was removed from the incubator and distributed evenly into 4 centrifuge bottles. These were spun in the centrifuge for 10 minutes at 4°C and 3000 X g.
2.2.2 Breakdown of *E. Coli* Membranes in preparation for Protein Purification

The resulting pellets from the 4 centrifuge bottles were dissolved in 30 mL PBS with 300 uL EDTA and 300 uL Protease Inhibitor (Thermo Scientific). This solution protects the protein from degradation at higher temperatures. Once the bacterial pellets were dissolved, the sample was sonicated to break down membranes within the bacterial cell to allow for the protein of interest to be isolated.

For sonication, the sample was originally placed in a styrofoam container with pieces of ice. However, it was determined through subsequent trials that the sample was heating too much using this method and this resulted in the protein breaking into fragments. Therefore, in future trials, an ice water bath was utilized. The ice water had better contact with the sample and prevented it from heating during sonication.

The sample was sonicated for a total of 3 minutes at an amplitude of 65% with a pulse of 5-sec, followed by a 25-sec pause. This was repeated twice, with a 5-10 minutes break between cycles to prevent overheating.

2.2.3 Isolation of VEGF-ELP

After sonication was used to break down the *E. coli* membranes, the sample was spun in a centrifuge at 4°C for 30 minutes at 15,000 X g to collect all the bacterial components into a pellet. Following this step, ITC was used to purify the protein from the other bacterial components. ITC involves alternating hot and cold centrifugation spins. At 40°C, the protein formed nanoparticles. This was further induced by adding sodium citrate to the sample. After the hot centrifuge spun for 15 minutes at 15,000 X g the protein was in the pellet and soluble contaminants were within the supernatant.
The pellet was then be dissolved in 7.0 pH PBS. Dissolution in the PBS was aided by cooling the sample on ice for 10-20 minutes. After the dissolution of the sample in the cold PBS, the sample was spun at 4°C for 10 minutes at 17,000 X g. As a result of this spin, the VEGF-ELP was localized to the supernatant and insoluble contaminants were localized to the pellet.

The hot and cold centrifugation spins were repeated two more times until all soluble and insoluble contaminants were removed and the protein was properly isolated from the other contaminants. A sample was taken from the supernatant after every spin for purity assessment. The concentration of the final sample was determined using a nanodrop and BCA Assay.

2.2.4 Assessment of VEGF-ELP purity

After each spin, a sample was taken from the supernatant. These samples were run through an SDS-PAGE gel. 19.5 uL of each sample was loaded into each well with 10.5 uL from a stock solution of loading buffer and β-mercaptoethanol. These samples were heated at 95°C for two minutes and loaded onto a NuPAGE 10-well gel (Thermofisher) which is used with the NOVEX system. All relevant reagents were obtained from NOVEX. The gel was run for 5 minutes at 50 mV and 45 minutes at 200 mV. The gel was then washed with autoclave water and washed for two hours with the stain.

SDS-PAGE gels were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories), blocked with blotting-grade blocker (Bio-Rad Laboratories). The membrane was washed with 7 mL of casein and 1.4 uL primary antibody, mouse anti-HIS. The membrane was washed with the antibody overnight. The following day, the membrane was washed with 7 mL of casein and the secondary antibody for an hour. The membrane was then washed for a minute and exposed to the TMB blotting solution, to reveal positive HIS-bands.
2.3. Results

2.3.1 Cloning and expression of VEGF-ELP

The plasmid consisting of 50 pentapeptide repeats of ELP and a single chain of VEGF is shown below:

Sequence for **Mph1103I-6xHIS-VEGF-3 x GAL linker - NdeI**
(498 bp - 166 amino acids - from NM_001287044.1)

```plaintext
atgCATCACCATCAACCATCAC

GCACCAGAAGCAGACTCACTCAGATCTCACTCAGACACACCTGGATGACTTTG

GCACCCATGGCACAGTCTCTTTGCTGAGCTATCTGTTTGTGCTGAGCTATCTGTTTGTAGCTATCTGTTTGTGCTGAGCTATCTGTTTGTAGCTATCTG

GGGCCGGGCGTGGGT

GTACCGGGCTGCGGT

GTTCCTGGTGTCGGC

GTGCCGGGCCCTGGCCG

TGA

TTG

Aagctt

CATATG
```

**ELP Motifs V40C2 Mph1103I-NdeI-ELP-HindIII**

```plaintext
atgCATCACCATCAACCATCAACCATCAACGTGGTGGTGAAGATCTCTGTATTTTCTAGGCACCTGGATGACTTTG

GCACCCATGGCACAGTCTCTTTGCTGAGCTATCTGTTTGTGCTGAGCTATCTGTTTGTAGCTATCTGTTTGTAGCTATCTGTTTGTAGCTATCTGTTT

GTACCGGGCTGCGGT

GTTCCTGGTGTCGGC

GTGCCGGGCCCTGGCCG

TGA

TTG

Aagctt

CATATG

GlyProGlyValGly ValProGlyValGly

GTTCCGGGCGTGGGT

GTACCGGGCTGCGGT

GTTCCTGGTGTCGGC

GTGCCGGGCCCTGGCCG

TGA

TTG

Aagctt

CATATG
2.3.2 Modifications to Growth and Purification After Early SDS-PAGE Results

Purification using ITC resulted in sufficient protein yield for experiments as determined by a BCA assay. Upon completion of three rounds of ITC purification, the purified sample was turning cloudy when held in hand (Figure 3), which indicated of the high concentration of ELP within the sample. SDS-PAGE and western blot experiments showed that the cycling process was effective in isolating the VEGF-ELP from the bacterial contaminants. However, early SDS-PAGE results revealed multiple bands corresponding with different proteins after the final spin (Figure 4). This indicated that the final protein was breaking into multiple fragments. Repeated tranformation and purification trials were performed to optimize the technique so that breakage did not occur.
One change that was made to the bacterial growth method was that the bacteria were not induced with IPTG. In earlier trials, the absorbance of the bacterial sample was measured after adding the LB sample to the 1 L of TB. When the sample's absorbance reached around 0.6 due to the bacterial growth, the batch was induced with 1 mL of IPTG and 5 mL of proline. However, it was later determined that this step was unnecessary and so it was removed from the protocol.

A change that was made in the purification technique in future trials was that the sample was not placed in the hot water bath at 40°C before the hot spin. Instead, it was determined that the salt was sufficient in inducing nanoparticle formation. This was indicated by the immediate transition from clear to milky cloudy following the addition of the salt to the sample. Additional heat could have been a reason that was contributing to the fragmentation of the protein, which was being observed in the SDS-PAGE results.
Another change that was made was that the sample was left on ice for longer periods of time before the cold spin to allow for best dissolution so that less protein would be lost in the pellet following the cold spin.

Minor changes were also made to the purification process to reduce the protein lost during each step. For example, fewer transitions were made between tubes and the dissolution of the protein in the PBS was improved by using a cut pipette tip.

### Figure 4. Early SDS-PAGE Results

Supernatant following the first hot spins contained soluble contaminants (1). Supernatant following the first cold spin indicates separation of VEGF-ELP from insoluble contaminants (2). Additional hot spins (3 and 5) and cold spins (4 and 6) removed residual soluble and insoluble contaminants. However, multiple bands were appearing in the C3 column, that corresponded with different molecular weight proteins. The transformation and purification processes were adjusted and fine-tuned to prevent the formation of multiple bands.

#### 2.3.3. SDS-PAGE and Western blot

After making the changes to the purification technique, the SDS-PAGE results revealed that there was no band separation (Figure 5). Supernatant following the 1st hot spin contained some level of soluble contaminants. Supernatant following the first cold spin indicated the separation of VEGF-ELP from insoluble contaminants. Additional hot spins and cold spins removed residual soluble and insoluble contaminants, respectively. The final hot spin and cold spin removed these
contaminants. The major band location observed in lane 6 is consistent with the theoretical VEGF-ELP molecular weight of 42.1 kDa.

Similarly, the western blot was conducted with antibodies that would specifically bind to the histidine tag portion of the protein. If the protein in the sample was not the protein of interest with specificity for the antibody, no band would appear in the western blot image. The western blot image revealed a band around the theoretical molecular weight of the protein, which indicates that the protein in the sample is the VEGF-ELP protein.

**Figure 5. Final SDS-PAGE and Western Blot Results.** The results show the characterization of VEGF-ELP after three rounds of ITC after making alterations to the purification process. Supernatant following the first hot spins contains some level of soluble contaminants (1). Supernatant following the first cold spin indicates separation of VEGF-ELP from insoluble contaminants (2). Additional hot spins (3 and 5) and cold spins (4 and 6) removed residual soluble and insoluble contaminants. The major band observed in (6) is consistent with theoretical molecular weight of VEGF-ELP which is 42.1 kDa. Right side panel shows a western blot image of purified VEGF-ELP, with an antibody staining for the HIS tag.
2.4 Discussion

In industry, protein purification is often done using chromatographic purification techniques. However, these methods can often result in a low yield and high cost in terms of labor and manufacturing. Therefore, one of the specifications for our novel treatment alternative was that it should be able to be easily purified and manufactured. This would make the clinical translation of the product more feasible and would greatly reduce the cost of the treatment.

As an alternative approach to protein purification, ITC was investigated. One of the benefits of using ELP as a fusion partner is that it reversibly forms nanoparticles above a transition temperature. This property of ELP makes it a good candidate for ITC purification. It can be easily purified using alternative hot and cold spins as shown in prior research studies [33, 34, 35]. For example, one study showed that inverse transition cycling of SDF1-ELP, with two alternating hot and cold spins, effectively removed all contaminants and resulted in the isolated protein [35]. Similarly, in our experiment, SDS-PAGE and western blot results verified that ITC was effective in isolating the VEGF-ELP from the bacterial components. As shown in the SDS-PAGE results, three rounds of alternating hot and cold spins were sufficient in removing all soluble and insoluble contaminants. This method efficiently provided a protein with relatively high yield and purity.

Ease of production will result in a lower cost. A cheaper treatment option with high efficacy would be a viable treatment option for people who may not be able to afford high-cost treatment options such as hyperbaric oxygen chambers and ointments that need to be reapplied frequently. Importantly, diabetic patients in third world countries will have a much higher incidence of chronic wounds and this protein has the potential to be a life-changing treatment for these people.
Chapter 3: Characterization of VEGF-ELP Nanoparticles

3.1 Introduction

In this chapter, we conducted a turbidity test to assess the nanoparticle formation property of the VEGF-ELP fusion protein. Formation of nanoparticles at a physiologically relevant temperature is essential to shield the VEGF from proteases, allowing it to remain active within the wound for longer periods, which is the premise of this thesis. Therefore, a turbidity test was required to show that nanoparticle formation does begin at around 37°C [37, 38].

Once it was clear that nanoparticles were forming as predicted, it was necessary to characterize these nanoparticles. This was done by using Dynamic Light Scattering (DLS) to determine the size of the nanoparticles at a particular concentration. This information will make it possible for the optimization of growth factor delivery in the future and will give a better understanding of how the protein will act in vivo [39,40].

3.2 Materials and Methods

3.2.1 Visual Assessment of Nanoparticle Formation

The turbidity of a 1 uM solution of VEGF-ELP was visually observed at 4°C and 40°C. Observations were made on a 1.2 mL sample in a cuvette that was removed from the Malvern Zetasizer machine at the specific temperatures. The cuvettes were held against a black board to better visualize the degree of turbidity.
3.2.2 Determination of Transition Temperature of VEGF-ELP

A 5 uM sample of the VEGF-ELP was placed into a 96-well plate in triplicates. The plate was then placed into a spectrophotometer plate reader and the absorbance was measured at a wavelength of 350 nm at an increasing temperature from 25-45°C. The temperature was increased at 2°C increments and there was a 5-minute delay before each temperature reading to accommodate for the delay between the machine’s temperature assessment and the sample’s actual temperature. This time delay also allowed nanoparticle formation to reach equilibrium.

3.2.3 Determination of particle size of VEGF-ELP nanoparticles

DLS was used to determine the particle size. This method measures the intensity in scattered light fluctuations as a result of the Brownian motion of the particles. The intensity is then used by the machine to calculate the particle diameter since random particle motion is dependent on particle size [39].

A 1 uM sample was loaded into the cuvette and placed into the Malvern Zetasizer. It was specified to the software that the particle being assessed was a protein and that it was suspended in PBS. Particle size was measured as 4°C and 37°C to get a comparison. It was expected that proper results would not be seen at 4°C because the protein is soluble at this temperature. At 37°C, we expected the protein to form the nanoparticles, of which the machine could assess the size.

3.3 Results

3.3.1 Turbidity Test

The samples removed from the Malvern Zetasizer were immediately placed against a black-board to observe the turbidity at the different temperatures. Visual observation revealed that the 1 uM
VEGF-ELP sample was clear at 4°C and cloudy at 37°C as seen in Figure 6B. When the cloudy solution was placed into an ice container, it became clear again within 20-30 seconds. This demonstrates that there is nanoparticle formation at 37°C and that this is a reversible formation.

This visual observation was also quantified by measuring the absorbance of the sample at increasing temperatures. When this experiment was conducted the first time, the delay between the plate reader measurement time and the time for the sample to heat and begin to form nanoparticles was not accounted for. This resulted in the temperature increasing very high without any absorbance change in the sample. The dashed line in Figure 6A shows the graph obtained, in which the temperature increases to 42°C before any observable increase in the absorbance. It was determined that this graph was not accurate because visually the sample started to become cloudy at 37°C.

Therefore, this experiment was repeated. The second time when the experiment was conducted, the delay was accounted for. A five-minute delay was placed between the time the plate reader reached a certain temperature and the measurement of the sample was taken. This delay time gave the sample enough time to heat up and for nanoparticles to begin to form. The solid line in Figure 6A shows the graph of absorbance verses temperature at increasing temperatures from 25°C to 45°C when the delay time was accounted for. As can be seen, the absorbance starts around 0.13 and begins to increase quite steeply at 37°C and continues to increase as the temperature increases. At 45°C, the absorbance of the sample is around 0.52. The rise in temperature at 37°C reveals that this is the transition temperature of the VEGF-ELP.
3.3.2 Nanoparticle Size

The dynamic light scattering experiment revealed a mean particle size of 835 nM as shown in Figure 7 and a polydispersity index of 0.253. The polydispersity index is a measure of the broadness of molecular weight distribution. The larger this value, the broader the molecular weights of the particles within the sample. Since this value is between 0.05-0.7, we can conclude that the size distribution of the nanoparticles is narrow (39).

Figure 7. DLS Analysis at 37°C. The size of the VEGF-ELP nanoparticles in a 5 uM sample was measured.
3.4 Discussion

After purification of the protein product, characterization was done using a turbidity test to assess the ability of the ELP to self-aggregate above a transition temperature. It was seen that at a temperature of 37°C, the absorbance of the VEGF-ELP solution began to increase at a steep rate, indicating that this was the temperature in which the proteins were beginning to aggregate into nanoparticles [37]. The formation of nanoparticles at a physiologically relevant temperature of 37°C allows us to hypothesize that the VEGF-ELP fusion protein will be more stable in the diabetic wound environment over native VEGF because the protein will be protected from degradation. Essentially, the fusion protein acts as a protected “drug depot” to release drug within the wound for extended periods of time, reaching better therapeutic efficiency [36].

Upon verifying that nanoparticles were forming at 37°C as predicted, DLS was used to determine the size of the nanoparticles to be ~850 nm. This quantification is important when assessing drug delivery since particle size affects the biodistribution of the particle within the wound, the colloidal stability of the particle, and the clearance of the nanoparticle from the wound environment. Research shows that colloidal stability tends to increase with increasing particle size until it reaches a maximum and then decreases as particle size increases [41].

The effect of particle size on wound healing was also investigated in one study by Nagai et al. In this research, it was found that the size of the nano dispersion of the drug rebamipide (REB) could be adjusted to optimize accumulation and therapeutic effect on corneal wound healing. Three nanoparticle sizes, including 735nm, 150nm, and 45 nm, were compared. It was found that the amount of REB instilled in the wound and the amount of corneal healing was highest for rats treated with the 150 nm dispersions [42]. This could be explained by the fact that nanoparticles that were too small had increased drug solubility and dissolved quickly into the
lacrimal fluid of the eye, losing their bio-activity. Particles that were too big, on the other hand, were unable to diffuse evenly throughout the wound and were colloidally unstable. As shown by this study, the size of the VEGF-ELP nanoparticle may need to be adjusted before clinical translation to better optimize delivery, particle stability, and overall therapeutic efficiency.
Chapter 4: VEGF-ELP Stimulates Proliferation of Endothelial Cells In Vitro

4.1 Introduction

In this chapter, an MTS proliferation assay was conducted to demonstrate that VEGF-ELP can enhance the proliferation of endothelial cells. Native VEGF plays an integral role in enhancing endothelial cellular proliferation by improving the proliferative capacity of the cells through mediation of the cell cycle, prevention of apoptosis by inducing expression of certain proteins, and maintenance of cell attachment through stimulation of fibronectin and integrins [25, 43,44]. Endothelial cells lay a framework for the granulation tissue and also significantly contribute to the formation of a new lumen to make the new capillary network within the wound [1,11,12]. Increased proliferation, as well as restoration of blood flow, are two factors that will allow for acceleration of healing of the chronic wound. Therefore, it is important to show that the VEGF-ELP fusion protein has the same biological activity and can enhance the proliferation of HUVEC cells to the same degree as native VEGF.

4.2 Materials and Methods

4.2.1 Standard Curve

A cell stock of 200,000 cells/mL was created. 1000-20,000 cells were seeded in a 96-well plate in triplicate in M200 media (Life technologies) with LSGS supplement overnight (Figure 8). For the standard curve, after 24 hours, the cells were exposed to MTS

Figure 8. Standard Curve Preparation. The plate shows a seeded standard curve plate with HUVEC cells seeded from 1000-20,000 in triplicate overnight after being stimulated with MTS cell titer for 24 hours of incubation. The blue arrow shows the wells seeded with 1,000 cells and the black arrow indicates the wells seeded with 20,000 cells.
cell titer (Promega) as per manufacturer instruction in M200 media and incubated for two hours. The absorbance was then measured at 492 nm on a spectrophotometer plate reader. A standard curve of cellular concentration versus absorbance was created. The equation of the line would be utilized to convert the absorbances of the experimental data to cellular proliferation fold change.

4.2.2 Experimental Conditions

HUVEC cells (5,000/per well) were seeded in a 96 well plate. These cells were initially seeded with M200 media with LSGS supplement overnight. After 24 hours, the cells were stimulated with M200+2% FBS in addition to 3 nM of either ELP, VEGF, or VEGF-ELP. The ELP group acts as a second control, to show that any results seen in the VEGF-ELP group were not due to the ELP protein alone. After 48 hours, the media was replaced and MTS cell titer was added to all wells. After two hours, the absorbance was measured at 492 nm and the data was converted to a fold change to see the difference among the groups.

4.3 Statistical Analysis

Data is presented as a mean ± SEM (n=9). Statistical analysis was done using a one-way ANOVA followed by a post hoc Tukey HSD test. A p-value <0.05 was considered as statistically significant.
4.4 Results

The data (Figure 9) shows that the proliferation presented as fold change for the control group and ELP group were 1.22 ± 0.12, and 1.40 ± 0.19, respectively. For the VEGF and VEGF-ELP group, the proliferation was 2.29 ± 0.25, and 2.37 ± 0.26, respectively. An ANOVA followed by a posthoc Tukey HSD test revealed a significant difference in fold change between the control and the VEGF/VEGF-ELP groups (p=0.003), indicating that VEGF/VEGF-ELP showed significantly greater proliferation over the control. Similarly, the VEGF/VEGF-ELP groups had significantly greater proliferation rates over the ELP group alone (p=0.04). No statistical difference was observed in proliferation between the VEGF and VEGF-ELP groups (p=0.16).

Figure 9. Proliferation of HUVEC cells. The graph shows proliferation presented as a fold change under different conditions. **p<0.01 indicates a comparison between control group and VEGF/VEGF-ELP groups while #p<0.05 indicates a comparison between ELP and VEGF/VEGF-ELP groups.
4.5 Discussion

Endothelial cells play a pivotal role in the healing of wounds. They lay the framework for the creation of the granulation tissue [1]. These cells are also responsible for creating a new lumen, which will form the capillaries of the new vascular network. Chronic wounds, as discussed in the introduction, often get stuck in the inflammatory stage of wound healing, which leads to inhibition of endothelial cell proliferation [14]. As a result, the creation of the granulation tissue and renewal of the vascular network is halted, preventing the wound from closing. Without a restoration of blood flow to the wound, many other processes such as immune response and cellular migration are also negatively affected due to limited delivery of essential nutrients such as oxygen [15,16].

Previous research has shown that native VEGF plays a key role in enhancing endothelial cell proliferation through a variety of receptor-mediated actions, such as mediating the cell cycle, preventing apoptosis, and by regulating cell attachment through interaction with integrins and vitronectin [25, 44]. In one study, VEGF was encapsulated in PEG and PCLA microspheres. The biological activity of the released growth factor from the microspheres was then assessed using an in vitro proliferation assay on HUVEC cells. The proliferation of the cells in the presence and absence of the microspheres was compared. Results showed that the VEGF encapsulated microspheres significantly increased the proliferation of the HUVEC cells, with a p<0.05 [43].

Therefore, research has shown that the exogenous application of native VEGF has been shown to enhance cellular proliferation. However, in vivo results suggest that the applied growth factor is quickly degraded within the body, rendering it therapeutically inefficient as a treatment option [31]. Therefore, the ability of VEGF-ELP to enhance cellular proliferation needs to be assessed to understand if this fusion protein can also undertake the responsibility of restoring endothelial cell proliferation within the chronic wound environment.
The results of this experiment showed that VEGF-ELP was able to enhance cellular proliferation of the endothelial cells comparably as native VEGF. The p-value < 0.05 which was obtained using an ANOVA revealed that VEGF and VEGF-ELP both had statistically greater proliferation when compared to the control groups with just the media and ELP alone. From these results, it can be concluded that the fusion of VEGF with the ELP component did not inhibit the biological activity of the growth factor in terms of enhancing endothelial cell proliferation. Thus, it can be hypothesized that in vivo the VEGF-ELP will be able to bind to the VEGFR2 receptor to enhance cellular proliferation and allow for the wound to progress past the second stage of wound healing.
Chapter 5: VEGF-ELP Enhances Cellular Migration of Fibroblasts In Vitro

5.1 Introduction

In this chapter, a fibroblast scratch assay was conducted to demonstrate that the addition of VEGF-ELP can enhance the migration of fibroblasts in vitro. Native VEGF increases fibroblast migration by promoting extracellular matrix synthesis, increasing secretion of fibronectin to create the extracellular matrix, and regulating various extracellular matrix proteins [25]. The migration of fibroblasts is a key process that occurs during the proliferative stage of wound healing [11,12]. It is imperative to show that VEGF-ELP can promote fibroblast migration to the same degree as native VEGF.

5.2 Materials and Methods

50,000 fibroblasts were seeded per well in a 24-well plate. The cells were initially seeded in Dulbecco’s Modified Eagle Medium (DMEM with 10% FBS+1% P/S) and incubated for 48 hours. Then cells were then stressed with base DMEM (without FBS) for the next 24 hours to prevent the cells from proliferating too fast. After 24 hours, each well was scratched and stimulated with different conditions in base DMEM (Gibco). The different conditions were the control, which was just base DMEM, 5 nM ELP, VEGF, and VEGF-ELP in base media. Images were captured for the next 2 days using a light microscope.
5.3 Statistical Analysis

The area of each scratch was measured using the ImageJ analysis tool (NIH). The areas of the scratch taken over the three days were converted to a % scratch closure using Equation 1 below.

\[
\% \text{ wound closure} = 100 \left( \frac{\text{New Scratch Area}}{\text{Initial Scratch Area}} \right) - 100 \tag{Equation 1}
\]

A graph was created showing the quantified data of wound closure (%) as a function of time. Data are presented as mean ± SEM (n=6). Statistical analysis was done using a one-way ANOVA followed by a post hoc Tukey HSD test. A p-value<0.05 is considered statistically significant.

5.4 Results

Images taken with the light microscope show that the scratch created for the VEGF-ELP group closed to the greatest degree compared with the VEGF, ELP, and control groups (Figure 10). The data was quantified and graphed as a percent wound closure (Figure 11). The scratch in the VEGF-ELP group closed to about 84 ± 4.0 %, the VEGF group to 74 ± 7.5%, the ELP group to 63 ± 4.2%, and the control group to about 64 ±1.1%. The VEGF-ELP group scratch closed to a significantly greater degree than the control (p<0.001) and ELP group (p<0.001). The VEGF group scratch closed to a significantly greater degree than the ELP group scratch (p=0.050). However, no statistical difference was observed between the VEGF and VEGF-ELP groups (p=0.231).
Figure 10. Light Microscope Scratch Images. Images were taken at 0 hr, 24 hr, and 48 hr at 10x magnification after scratching and stimulating cells.

Figure 11. % Wound Closure under Different Conditions. Graph shows the quantified data of scratch closure (%) as a function of time. Data are presented as mean ± SEM (n=6). Statistical analysis was done using a one-way ANOVA followed by the post hoc Tukey HSD test. \(^{^p}<0.01\) and \(^{^{^p}}<0.001\) indicates statistical significance between the VEGF-ELP and control groups. \(^{##}p<0.01\) and \(^{###}p<0.001\) indicates statistical significance between the VEGF-ELP and ELP groups. *\(p<0.05\) indicates statistical significance between the VEGF and ELP groups.
5.5 Discussion

In this experiment, the goal was to show that VEGF-ELP enhances fibroblast migration to a similar extent as native VEGF. If VEGF-ELP can show the same biological activity as VEGF with the additional bonus of remaining therapeutically active for longer periods of time, it could be valuable as a novel treatment option for chronic wounds. Therefore, we compared the fibroblast migration in the presence of VEGF and VEGF-ELP, as well as to the control and ELP alone. Fibroblast migration was assessed by creating a scratch and recording the percent scratch closure over two days. The results revealed that the VEGF-ELP treated scratch closed to about 84% in two days, whereas the control and ELP treated scratches only closed to only 64% and 63% respectively. The p-value<0.05 for VEGF-ELP compared to the control and ELP groups indicated that the VEGF-ELP protein statistically increased fibroblast migration.

Migration of fibroblasts is a key process that occurs during the proliferative stage of wound healing, along with endothelial cell proliferation [1,11,12]. Migration of fibroblasts is imperative for wound closure and it is one of the main steps that allow for collagen remodeling and wound contracture during the remodeling phase of wound healing. VEGF plays a direct role in increasing fibroblast migration by promoting extracellular matrix synthesis, increasing leakage of fibronectin to create the extracellular matrix, and regulating various extracellular matrix proteins [25,26, 44].

As mentioned, migration of the cells is heavily influenced by the interactions of the cell with the extracellular matrix. One study conducted by Byzova et al. found that VEGF plays a pivotal role in the activation of key extracellular proteins such as integrins. Binding of VEGF to the VEGFR2 receptor activates the avb3 integrin [44]. In turn, activation of this integrin increases receptor-ligand binding of the cell. Enhanced cellular adhesion within the extracellular matrix leads to increased migratory capacity.
Another study, conducted by Atsushi et al, suggested that VEGF could also play a role in how cells migrate, in addition to their rate of migration. In this study, the role of VEGF and bFGF on the migration of endothelial cells was investigated using a checkerboard migration assay [45]. Their primary result was that VEGF can enhance cellular migration to a greater degree than bFGF. A secondary result was then the manner of migration within the assay was varied for the cells exposed to VEGF and bFGF. Cells growing in the presence of bFGF showed a chemokinetic migratory pattern, in which the cells randomly migrated in many directions. In contrast, cells grown in the presence of VEGF showed a chemotactic migratory pattern, in which cells migrated in a certain direction from where there was a lower concentration of the VEGF to where there was a higher concentration [45]. This research provides evidence that VEGF can be used as a powerful tool to enhance cellular migration and accelerate wound closure. Knowledge about the way VEGF affects the rate and manner of cellular migration can be studied in future experimentation to fine-tune the application of the growth factor to optimize healing.

The results obtained in this experiment suggest that exogenous application of VEGF-ELP to a chronic wound would directly enhance the migration of fibroblasts, accelerating the time to wound closure. Studies in vivo will provide more evidence on the biological activity of the protein and its effect on the different types of cells that play a role in the remodeling of injured tissue.
Chapter 6: VEGF-ELP Increases Angiogenesis *in Vitro*

6.1 Introduction

In this chapter, we performed an angiogenic tube assay to demonstrate that VEGF-ELP can stimulate angiogenesis by showing that VEGF-ELP increases the formation of capillary-like tubes *in vitro*. Native VEGF binds to the VEGF-R2 receptor on endothelial cells, initiating a key cascade that results in increased capillary tube formation [25, 31]. These cells synthesize the lumen needed for the tubes that form the vascular network, leading to increased angiogenesis [8, 9].

As discussed previously, research shows that chronic wounds have impaired oxygen flow due to insufficient angiogenic response to hypoxia. Therefore, restoring the vascular perfusion of the wound is imperative for restoring the delivery of oxygen to the wound so that the cells have the required energy to move into the proliferative stage of wound healing [8, 9, 10].

As a result, increased tube formation is essential for the healing process and this assay provides a useful tool for assessing the angiogenic potential of the fusion protein. Specifically, this experiment aims to show that VEGF-ELP can enhance capillary tube formation in a similar method as native VEGF is known to do.

6.2 Materials and Methods

Human umbilical vascular endothelial cells were plated (120,000 per well) in a 24-well plate. The cells were cultured in PRF+ M200 media. They were then seeded on Matrigel (Corning Life Sciences) and exposed to the different stimuli in different groups for ~18 hours. The groups were a control group with M200-PRF media, control + with M200-PRF media with LSGS supplement, M200-PRF media with 500 nm ELP, and M200-PRF media with 3 uM VEGF and VEGF-ELP.
Then, cells were stained with calcein (8 μg/mL in M200-PRF media). Images were captured under a fluorescent microscope.

### 6.3 Statistical Analysis

The number of dots, tubes, and meshes were counted using ImageJ software (NIH) and presented as a mean ± SEM (n=3). Statistical analysis was done using one-way ANOVA followed by post hoc Tukey HSD test. A p-value<0.05 was considered as statistically significant.

### 6.4 Results

As seen in Figure 12, the control and ELP groups revealed little to no tube or mesh formation. The control positive group had minimal tube formation. The VEGF and VEGF-ELP groups showed significant tube and mesh formation.

The number of dots, tubes, and meshes in each image were quantified using Image J (Figure 12). There were about 28 ± 1, 40 ± 1, 30 ± 5, and 34 ± 1 dots in the control, control +, ELP, and VEGF groups respectively and 50 ± 1 dots in the VEGF-ELP group. The dots represent the cells within the Matrigel. Quantification of the number of cells in each image gave an idea as to how the cells were proliferating in the Matrigel. The one-way ANOVA revealed that VEGF-ELP had a greater cell count over the control (p<0.001).

There were about 15 ± 2, 47 ± 13, and 7 ± 1 tubes in the control, control +, and ELP groups respectively and 63 ± 7 and 57 ± 4 tubes in the VEGF and VEGF-ELP groups. Statistical analysis results showed that the VEGF and VEGF-ELP groups had statistically greater tube formation over the control group (p<0.01, p<0.05).
The number of meshes were also quantified within the Matrigel to assess the formation of tubular connections. These connections would characterize the formation of vascular networks *in vivo*, which would allow for enhanced perfusion of the wound and would enhance nutrient delivery, thereby accelerating wound closure. 5 ± 2, 25 ± 4, and 2 ± 1 meshes were counted in the control, control +, and ELP groups respectively. Around 31 ± 1 and 36 ± 2 meshes were counted in the VEGF and VEGF-ELP groups respectively. An ANOVA (p<0.001) revealed that the VEGF and VEGF-ELP groups had statistically greater mesh formation over the control. This indicates that the native VEGF and VEGF-ELP proteins would be able to enhance vascular perfusion within a wound.

![Figure 12. HUVEC Tube Assay Results.](image)

Representative images of tube formation are shown in different groups. Images were captured with a fluorescent microscope and the number of dots, tubes, and meshes were counted using Image J analysis software. The lower panel shows the quantified data of the number of dots, tubes, and meshes. *p<0.05, **p<0.01, and ***P<0.001.
6.5 Discussion

Prior research has shown that VEGF does play a pivotal role in the formation of new vascular networks. Numerous studies have shown that hypoxia, or limited oxygen supply, is a stimulator for the release of VEGF, which in turn assists in stimulating angiogenesis [26, 46, 47]. However, in the chronic wound, hypoxia is unable to stimulate an angiogenic response that meets the nutritional needs of the cells [14, 15]. As a result, key cellular processes such as proliferation and migration are inhibited and the wound is unable to heal. Therefore, exogenous application of this essential growth factor could restore the angiogenic process, enabling an influx of oxygen which in turn would restore the wound healing process. Enhancement of the vascular network within the chronic wound is thus essential for treatment [25, 26].

Although previous studies have shown that exogenously applied VEGF is sufficient for stimulating angiogenesis, the quick degradation has tendered VEGF an impractical and costly option for restoring oxygen delivery to the wound [31]. Other methods, such as the use of the hyperbaric oxygen chamber, have been more widely used in the treatment of stubborn chronic wounds as an option for increasing the oxygen delivery. However, as discussed in the introduction, these chambers are often ineffective and can lead to a host of serious complications [23, 24]. Therefore, VEGF-ELP was investigated as a more practical option for increasing local angiogenesis and increasing oxygen delivery.

In this study, the objective was to show that VEGF-ELP can stimulate angiogenesis to the same degree or better when compared to native VEGF. Since the assay contained no proteases, we expected comparable results between the proteins if VEGF-ELP presented as biologically active.

Tube formation was assessed by considering the number of dots, tubes, and meshes within each image. Dots corresponded with the number of cells within the matrix and gave an idea as to
the level of proliferation of the cells. Tubes corresponded with the number of capillary shoots being created by the cells and gave quantified the relative amounts of the new lumen being synthesized by the cells. Meshes corresponded to the number of tubular networks between the cells. The results showed that the VEGF and VEGF-ELP both presented significantly increased endothelial cell tube formation when compared to the control groups, with just the media and just the ELP alone. This was seen through a higher number of the dot, tube, and mesh counts.

These results show that VEGF-ELP has comparable biological activity as native VEGF when it comes to stimulating angiogenesis. This allows us to hypothesize that VEGF-ELP will play a key role in stimulating angiogenesis when exogenously applied to a chronic wound. It should also have the key benefit of remaining therapeutically active for longer durations, thereby allowing for the restoration of vascular flow to the chronic wound after a few applications. Although the preliminary data presented thus far does suggest that VEGF-ELP does have profound biological activity, more evidence is required before it could be translated to clinical application.

Future studies may need to take into account other factors involved in angiogenesis and restoration of the vascular network. For example, in addition to counting the increase in tube formation, experimentation could be done to assess the increase in the volume of the tubular network. Larger tubes may allow for greater blood flow to the wound [48, 49]. One study conducted by Nakatsu et al investigated the effect VEGF concentration has on vessel diameter. It was found that varying the concentration of VEGF available to growing vessels, affects the vessel diameter. In the presence of large amounts of VEGF, vessel count and diameter were found to be greatly increased [48]. It was explained that this in part could be due to greater endothelial cell proliferation. This was seen in our study as a larger dot count in the presence of VEGF and VEGF-ELP. Increased cellular proliferation can lead to local mechanical forces on the cells. If the cells
are proliferating rapidly, the cells may form strong connections with neighboring cells and begin
to flatten the matrix [48]. Thus, the vessel has more area to grow circumferentially as opposed to
longitudinally. The diameter of the tubes within a network can play a large role in the delivery of
blood to the tissue. Thus, this may be a factor that should be investigated further in future
experiments.

Another consideration of enhancing angiogenesis using VEGF is that certain factors may
allow for increased stabilization of the neovessels. It has been found that introduction of fibroblast-
derived factors, such as Angiopoietin-1 in the assay with the endothelial cells leads to increased
sprouting, lumen formation, and tubular stability [48, 49, 50]. This could prove to be a tactic for
accelerating the restoration of the vascular network within the chronic wound and also for
stabilizing the new vascular networks to prevent adverse side effects such as tumor formation.
Perhaps a combination of different factors along with VEGF could be used clinically to accelerate
wound healing to the greatest degree, while preventing adverse side effects.
Chapter 7: Conclusion

7.1 Key Findings

Previous research conducted on the topical application of VEGF for chronic wound healing has demonstrated the ability to promote healing in experimental animals. However, the therapeutic efficiency of this protein is only achieved upon using high and frequent dosages due to the quick degradation of the protein \textit{in vivo} by proteases [31]. Besides, the purification of large amounts of protein through chromatographic methods can be a tedious and expensive process. This has rendered it difficult for the translation of VEGF as a clinical treatment for chronic wounds due to the impracticality as well as high cost.

Therefore, the goal of this research was to create a VEGF derivative that would have a similar biological effect as native VEGF, while resisting protease degradation and requiring a non-chromatographic purification process that would reduce manufacturing costs. Due to the requirements of the project, ELP was chosen as the best option as a fusion partner for VEGF. ELP proteins are being increasingly investigated in biomedical research due to their tendency to self assemble into nanoparticles above a transition temperature. This phase-transition property of ELP made it an ideal fusion partner for the VEGF because the formation of nanoparticles would enable the ELP to resist protease degradation \textit{in vivo} and would also make it easy to purify using a thermally driven centrifugation process [36].

To conclude, in this experimental study we successfully generated and isolated a fusion protein with VEGF and ELP domains. SDS-PAGE and Western blotting were conducted to show that protein could be easily and inexpensively purified using ITC. DLS experiments in conjugation with turbidity testing revealed that the fusion protein reversibly forms nanoparticles of \textasciitilde850 nm
in size above the transition temperature of 37°C. \textit{In vitro} experimentation with the proliferation, migration, and angiogenesis assays revealed that VEGF-ELP has a comparable biological activity to native VEGF on endothelial cells and fibroblasts. Together, these results allow us to hypothesize that VEGF-ELP will be able to aid in wound healing and will remain therapeutically active for longer periods of time than native VEGF without the need for frequent reapplication of high dosages. This research offers support that VEGF-ELP should be further investigated as a clinical treatment option to augment the healing of chronic wounds.

**7.2 Limitations and Future Steps**

**7.2.1 Monomer activity comparable to Nanoparticle Activity**

For better characterization of the therapeutic efficiency of the nanoparticle, it would be important to isolate the monomer activity \textit{in vitro} from the nanoparticle activity. The assumption was made that since the assays were kept at 37°C, the majority of the VEGF-ELP was in nanoparticle form. However, more realistically, a proportion of the protein would be in monomer form throughout the experiment. It would be important for better insight into the biological activity of the VEGF-ELP nanoparticle, to know how much protein was in the monomer form relative to the nanoparticle form.

In a previous study on the characterization of SDF1-ELP, an \textit{in vitro} intracellular calcium release assay was used to quantify the relative amounts of activity from the monomeric form relative to the nanoparticle form [36]. This was done by separating the freshly aggregated SDF1-ELP using a 10 nm pore membrane and comparing the calcium rise from the filtrate with the calcium rise from the material on the top of the membrane. In that study, a higher calcium release
was observed from the material at the top of the membrane relative to the filtrate, showing that the activity of the nanoparticles was greater. A similar study, in this case, could reveal the relative biological activity of the monomeric VEGF-ELP verses the nanoparticle form.

### 7.2.2 Stability Study

One of the main hypotheses presented in this research is that VEGF-ELP will be able to remain therapeutically active within the wound for longer periods of time without quickly being degraded by proteases. This hypothesis can be tested in a future study through both *in vitro* and *in vivo* experiments.

To test this hypothesis *in vitro*, a stability study should be performed to assess how much longer the VEGF-ELP remains active in the presence of a protease, relative to native VEGF. This could be done by mixing the VEGF-ELP and VEGF with a protease, such as elastase, and detecting the presence of the protein for a certain number of days. If the hypothesis holds, it would be expected that the VEGF-ELP would be detected in the assay for a longer duration of time than the native VEGF. This would mean that the VEGF-ELP is resisting degradation by the elastase and is, therefore, remaining in a solution for a longer time.

### 7.2.3 *In Vivo* Study

For more evidence on the therapeutic potential of the VEGF-ELP as a topical cream for chronic wounds, the protein should be tested in an *in vivo* model. Diabetic mice or spinal cord injury mice could be used, due to the impaired healing of their wounds [51, 52]. VEGF-ELP and native VEGF could be tested on a sample of mice with a biopsy taken from their dorsal side. Healing of the wound in the presence of the proteins would be assessed over a few weeks and
compared. This study would give more insight into whether VEGF-ELP could provide greater therapeutic efficiency than native VEGF and whether translation to the clinic is feasible.

### 7.3 Conclusion

Previous research on the applications of native VEGF for wound healing fails to consider that the need for high dosages and frequent applications render it an impractical treatment option for clinical use. As a result, clinicians often turn to other methods of treating chronic wounds. Many of these methods have shown to be ineffective or have negative side effects when used excessively. This research provides preliminary evidence that VEGF-ELP fusion proteins provide a potential treatment option for chronic wounds that is efficient, practical, cost-effective, and could augment the currently available treatments. Results have shown that this protein can be easily isolated using ITC and that it reversibly forms nanoparticles above a physiologically relevant temperature, which should allow it to resist protease degradation. *In vitro* experiments have provided evidence that the protein will enhance cellular proliferation, fibroblast migration, and tube formation, which are three key cellular processes that occur during wound healing. Together, these results suggest that VEGF-ELP could be a viable option for the treatment of chronic wounds. Further investigation should be done to assess the stability and activity of the protein *in vivo*. Once proven to have enhanced stability and *in vivo* efficacy, translation of this protein into clinical use could offer a cost effective, practical treatment option for the millions of people around the world who are affected by chronic wounds each year.
## 7.4 References


